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J. M. Martinez-Reyna
University Autonoma Agraria Antonio Narro

Kenneth P. Vogel
University of Nebraska-Lincoln, kvogel1@unl.edu

Carol Caha
University of Nebraska-Lincoln, caha@unl.edu

Donald J. Lee
University of Nebraska-Lincoln, dlee1@unl.edu

CELL BIOLOGY & MOLECULAR GENETICS

Meiotic Stability, Chloroplast DNA Polymorphisms, and Morphological Traits of Upland × Lowland Switchgrass Reciprocal Hybrids

J. M. Martínez-Reyna, K. P. Vogel,* Carol Caha, and Donald J. Lee

ABSTRACT

Switchgrass (*Panicum virgatum* L.) has two cytotypes or cytoplasm types, L and U, that are associated with the lowland and upland ecotypes, respectively. The L cytotypes are tetraploids while the U cytotypes can be either tetraploids or octaploids. The objective of this research was to characterize meiotic stability of reciprocal crosses of U and L plants as indicated by chromosome pairing at meiosis and to determine the mode of inheritance of chloroplast DNA (cpDNA) in the hybrids of these cytotypes. Morphological markers that characterize the parents and hybrids also were investigated to confirm that progeny were true hybrids. Reciprocal crosses were made between Kanlow (L tetraploid) and Summer (U tetraploid) plants. Pubescence on the upper surface of the leaf blade, foliage color, and seed size were evaluated as markers to verify hybridization. Meiotic pairing of some of the hybrids was analyzed at the diakinesis stage of meiosis by means of immature anthers. The clone pRR12 from a spinach (*Spinacia oleracea* L.) cpDNA library was used as a chloroplast hybridization probe to determine chloroplast inheritance. For all the morphological traits evaluated, the hybrids were intermediate in comparison to the parents except for seed width. Chromosome pairing was primarily bivalent in all hybrids. The viability of the hybrid seed and the normal meiotic chromosome pairing of the hybrids indicate a high degree of similarity between upland and lowland genomes. In the cpDNA analysis, all verified hybrids examined carried a fragment identical in size to the fragment of the female parent, indicating predominance of maternal inheritance of the cpDNA in switchgrass.

Switchgrass is a North American native, C4, perennial grass with an adaptation zone from Canada to Central America and from Nevada to the Atlantic Coast (Hitchcock, 1971). This cross-pollinated grass has been used for warm-season pasture and to reseed rangelands (Moser and Vogel, 1995). Recently the U.S. Department of Energy has identified this species as a promising biomass fuel crop (Lynd et al., 1991; Vogel, 1996).

Morphologically switchgrass is classified into lowland and upland ecotypes (Brunken and Estes,1975; Porter, 1966). Porter (1966) reported that the lowland plants in central Oklahoma were entirely tetraploid, whereas the upland plants were both hexaploids and octaploids. Barnett and Carver (1967) also reported the same ploidy pattern in plants from Oklahoma and Kansas. The lowland type has coarse and erect stems, glabrous leaves, and rust (*Puccinia graminis* Pers.:Pers.) resistance, and grows as a 0.6- to 3.0-m-tall semi-bunchgrass. The upland type has fine and semi-decumbent stems, pubescence in the upper surface of the leaf blade, short rhizomes which produce a sod, and less robust growth with a height of 0.9 to 1.5 m (Porter, 1966; Barnett and Carver, 1967). Recent analyses of lowland plants confirms that they are tetraploid ($2n = 4x = 36$) while upland plants are either tetraploids or octaploids (Hopkins et al., 1996; Hultquist et al., 1996).

The first molecular genetic basis for classification of switchgrass was provided by Hultquist et al. (1996). They surveyed 18 switchgrass cultivars and experimental populations for cpDNA polymorphisms by using four restriction endonucleases and 20 sorghum [*Sorghum bicolor* (L.) Moench] cpDNA probes and detected one polymorphism that was associated with the lowland-upland classification. The lowland type has a restriction site change that is not present in upland type. The enzyme/probe combination that detected the polymorphism was *Bam*HI/pLD5. The cytotypes were named as U and L after upland and lowland ecotypes, respectively. Hulquist et al. (1996) suggested that the cpDNA polymorphism found in upland and lowland ecotypes could be used to trace the mode of inheritance of the cpDNA in switchgrass which was not known. Gunter et al. (1996) assessed the genetic diversity among 14 switchgrass cultivars using RAPD markers. Cluster analyses of 92 polymorphic loci separated the population into two groups that matched the ecotypic classification and the cytotypic designation of Hultquist et al. (1996). These findings support the idea of Barnett and Carver (1967) and Brunken and Estes (1975) that the two ecotypes of switchgrass represent genetically distinct populations.

The meiotic chromosome pairing behavior of switchgrass has been studied by several authors. Barnett and Carver (1967) found that bivalent pairing in switchgrass is more nearly complete in tetraploids than in higher polyploids. Univalents occur in all ploidy groups, but in a smaller proportion of the total complement in tetraploids than in plants with higher ploidy levels. Trivalents and quadrivalents were not observed in tetraploids, while low frequencies of such configurations occurred

J.M. Martínez-Reyna, Univ. Autónoma Agraria Antonio Narro, Buenavista, Saltillo, Coah. México; K.P. Vogel, USDA-ARS, 344 Keim Hall, Univ. of Nebraska, P.O. Box 830937, Lincoln, NE; Carol Caha and D.J. Lee, Agronomy Dep., Univ. of Nebraska, Lincoln, NE 68583-0915. The reported research is from a dissertation submitted by the senior author (J.M. Martínez-Reyna) in partial fulfillment of the requirements for a Ph.D. degree at Univ. of Nebraska, where the senior author was financially supported by CONACYT-México. Journal Series No. 13019. Nebraska Agric. Exp. Stn. The research was funded in part by the U.S. Dep. of Energy's Bioenergy Feedstock Development Program at Oak Ridge National Laboratory, USDA-ARS, and the Univ. of Nebraska. Received 26 June 2000. *Corresponding author (kpv@unlserve.unl.edu).

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in octaploids and aneuploids. Brunken and Estes (1975) observed that in tetraploids the meiosis was normal and no multivalent associations were found. Lu et al. (1998) reported that primarily bivalent pairing was observed in meiosis of both tetraploid and octaploid cultivars; only occasionally univalent and multivalent associations were found. These observations seem to indicate that either both tetraploids and octaploids are disomic polyploids or that they are polysomic polyploids under strong selection for bivalent pairing. Until recently (Martínez-Reyna and Vogel, 1998) no fertile progeny had been produced by controlled crosses between upland and lowland cytotypes of switchgrass.

The objectives of this research were to characterize and study the meiotic stability of reciprocal hybrids between tetraploid lowland and tetraploid upland ecotypes of switchgrass as indicated by chromosome pairing at meiosis, to determine the mode of inheritance of cpDNA in switchgrass, and to identify morphological markers that distinguish hybrid plants.

MATERIALS AND METHODS

Plants from the cultivars Kanlow (K) and Summer (S) were used in this study. Kanlow is a lowland cultivar developed from 200 plants collected from a site near Wetumka, OK (Alderson and Sharp, 1955). Summer is a cultivar developed at South Dakota from a collection made near Nebraska City, NE (Alderson and Sharp, 1955). The cultivars Kanlow and Summer were classified as lowland and upland cytotypes respectively by Hultquist et al. (1996) and were designated as lowland and upland ecotypes based on random amplified polymorphic DNA (RAPD) markers by Gunter et al. (1996). Riley and Vogel (1982) reported both cultivars as tetraploids ($2n = 4x = 36$). Parent plants were identified by "K" or "S" for Kanlow or Summer, respectively followed by a clone number, e.g., K1 and S7. Progeny were identified as female clone × male clone, e.g., K1 × S7.

Controlled reciprocal crosses were made between Kanlow and Summer plants by the technique described by Martínez-Reyna and Vogel (1998). The progeny of three direct and reciprocal crosses and five of six parent plants were used in this study. One of the Summer parent plants died during the dormant period after seed harvest.

The F_1 hybrid seed was wet chilled at 5°C for 3 wk and germinated in super-cell cone-tainers (Steuwe and Sons, Corvallis, OR)[1] or minipots filled with a mixture of soil, peat and vermiculite (2-1-1 v/v/v) in a greenhouse with an 18-h photoperiod and a mean temperature of 28°C. The F_1 hybrid seedlings were transplanted into pots after 60 d of growing in cone-tainers and kept in the greenhouse. A solution of 4.5 g L^{-1} of water-soluble fertilizer (20-20-20) was applied every 30 d during the growing season.

To verify that progeny from reciprocal crosses were hybrids, the following morphological traits were screened in parent and hybrid plants: pubescence in the upper base of the leaf blade, plant color, and seed size. Pubescence at the base of the upper leaf blade was scored as a presence or absence trait at the seedling stage, foliage color varied from green to blue-green and was scored at heading stage, and seed size (length and width of mature caryopsis enclosed by lemma and palea) was measured for 15 seeds taken randomly from a set of seed obtained by bulking seed from four ripe open-pollinated panicles of individual plants. Mean comparisons of seed length and seed width among progenies and parents were done by *t*-tests.

Meiotic pairing in some of the hybrids was analyzed at the diakinesis stage of meiosis by means of immature anthers. Anthers were fixed in a 3:1 95% (v/v) ethanol:glacial acetic acid solution, stored at room temperature, and stained and prepared for observation using the acetocarmine squash procedures (Smith, 1947). The slides were observed under high magnification (×1000) of a light microscope (A.O. Spencer, American Optical Corp., Del Mar, CA). Digital photographs were taken by a camera (CCD Color Camera, VPC-920) connected to the microscope and printed with a digital printer (Mavigraph UP-1200A, Sony Corp.).

For DNA analyses, young leaf tissue was collected and lyophilized. DNA was extracted from lyophilized (300–400 mg) tissue by a hexadecyltrimethylammonium bromide (CTAB) procedure (Saghai-Maroof et al., 1984). The restriction enzyme *Bam*HI was used to prepare single digests of total DNA samples. Separate aliquots of total DNA (5 mg) were digested to completion under the conditions recommended by the enzyme suppliers. Digested DNA was loaded onto horizontal 0.8% (w/v) agarose gels and electrophoresed at 35 V for 16 h in TRIS-borate buffer (Sambrook et al., 1989). A lane of *Hin*dIII-digested lambda DNA was also loaded onto each gel to provide molecular weight markers. Gels were stained with ethidium bromide and photographed on a UV transilluminator (315 nm). DNA was transferred from gels onto nylon membrane by a neutral transfer Southern blotting method (Reed and Mann, 1985).

The sorghum probe pLD5 used by Hultquist et al. (1996) has been difficult to maintain. Hence, a restriction fragment subcloned from a spinach cpDNA library (Zurawski et al., 1981) was obtained for this study from the laboratory of Dr. Hans Bohnert, Dep. of Biochemistry, University of Arizona. This cpDNA clone, pRR12, spanned the homologous region of the sorghum clone pLD5 and detected the *Bam*HI restriction fragment polymorphism that differentiated upland and lowland switchgrass cultivars. The clone pRR12 has the 2KB *Eco*RI–*Bam*HI digest fragment of spinach chloroplast DNA containing *rbcL* which encodes the large subunit of ribulose

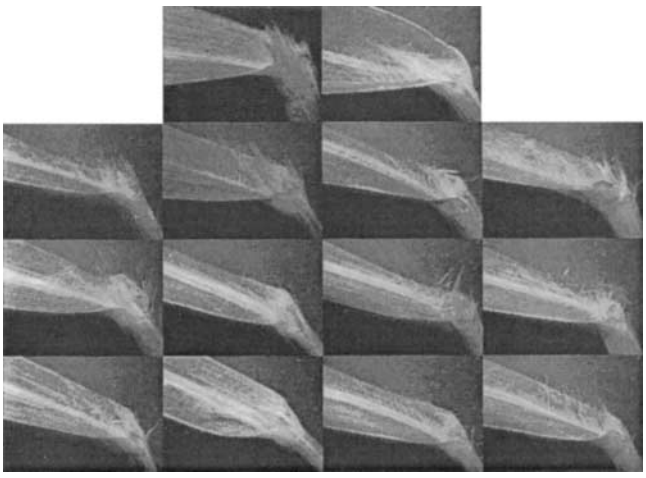

Fig. 1. Pubescence in the upper base of the leaf blade of parents and progenies of a female lowland and a male upland switchgrass hybridization. The parents Kanlow (female, left) and Summer (male, right) are shown in the top row and the progeny in the subsequent rows. The Summer (female) and Kanlow (male) cross produced similar results (not shown).

[1] Mention of a trade name does not constitute a guarantee of the product by the USDA or the Univ. of Nebraska and does not imply its approval to the exclusion of other suitable products.

Table 1. Seed length means comparisons (mm) for parent Kanlow (K) and Summer (S) plants and for their hybrids' progeny and associated *t* tests assuming unequal variances. *N* = 45 for parents and *n* = 105 for seeds from their hybrids' progeny.

Genotype	Mean	Standard deviation	SE	*t*	*P* > *t*
Kanlow	2.86	0.08	0.01		
Summer	2.44	0.12	0.01	19.11†	0.01**
K × S	2.59	0.11	0.01	14.59†	0.01**
S × K	2.65	0.10	0.009	12.53†	0.01**
K × S	2.59	0.11	0.01	6.93‡	0.01**
S × K	2.65	0.10	0.009	−10.73‡	0.01**
K × S	2.59	0.11	0.01	−3.92§	0.01**

** *P* significant at 0.01.
† Mean comparison with Kanlow.
‡ Mean comparison with Summer.
§ Mean comparison with S × K.

1,5-biphosphate carboxylase. This fragment includes the *rbcL* promoter, ribosome binding site, and terminator. The probe was labeled with digoxigenin-11-dUTP by random priming (Feinberg and Vogelstein, 1983). Digoxigenin-11-dUTP labeled probe hybridized to membrane-bound DNA was detected with an anti-DIG-alkaline phosphatase conjugate and a chemiluminescent substrate (Lumi-Phos 530, Roche Molecular Biochemicals, Indianapolis, IN). Prehybridization, hybridization, filter washing, chemiluminescent detection of hybridized membrane-bound fragments and membrane stripping after chemiluminescent detection were done following the procedures described by Muza et al., 1995.

RESULTS AND DISCUSSION

All Summer (S) parent plants were pubescent and green with the upper surface of the leaf blade appearing slightly blue-green. All Kanlow (K) parent plants were glabrous and blue-green in color. Both direct and reciprocal hybrids were intermediate to parents in pubescence (Fig. 1) and light green with the upper surface of the blade appearing blue-green. Hybrid plants appeared to show the green and blue-green colors of Summer but the blue-green color in the upper surface of the blade appeared quite similar to the blue-green color of Kanlow. Since plant color is a subjective trait, pubescence in the base of the leaf blade can be used as a distinctive marker to identify hybrids when Kanlow is the female parent and Summer the male parent. Kanlow seedlings from self-pollination will be glabrous.

All hybrids produced seed. The mean seed lengths of Kanlow and Summer were 2.86 mm and 2.44 mm respectively. Mean length of seed of the K × S and S × K F_1 hybrids were 2.59 mm and 2.65 mm, respectively. The mean length of seed produced by parents and hybrids were significantly different at a >0.01 in all combinations of two means on the basis of a two-tailed *t* test (Table 1). Mean width of seed produced by Kanlow and K × S hybrid plants were not statistically different nor were the mean seed widths of Summer and S × K hybrids indicating a possible maternal parent effect on seed size. All other mean comparisons were significantly different at $\alpha = 0.01$ (Table 2). The shape of seed produced by the hybrids was intermediate between the parents' seed shape (Fig. 2). For the morphological traits evaluated except for seed width, the hybrids were intermediate in comparison to the parents. Since all progeny regardless of the direction of the cross had pubescence on the upper leaf surface, leaf pubescence appears to be a dominant trait that is regulated by few genes.

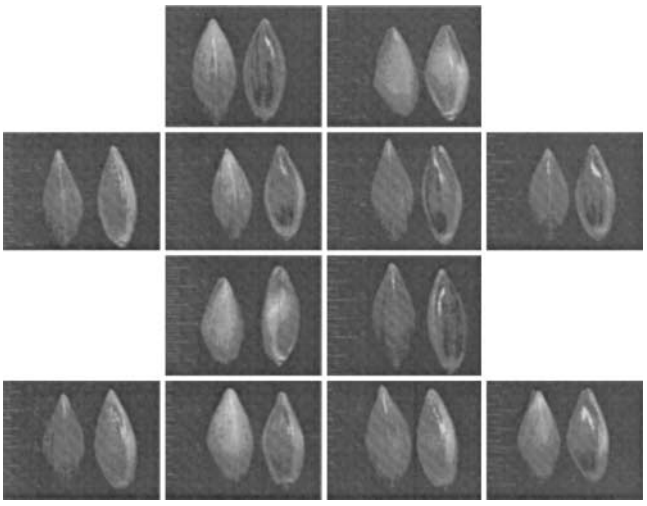

Fig. 2. Shape of seed of lowland by upland direct and reciprocal crosses. Top row: Kanlow (female, left), Summer (male, right); second row: progeny from the direct cross; third row: Summer (female, left), Kanlow (male, right); and fourth row: progeny from the reciprocal cross.

Meiotic pairing was studied using a total of 162 microsporocytes from three K × S hybrids and two S × K hybrids (Table 3). The chromosome pairing, analyzed at diakinesis stage of meiosis, was primarily bivalent in all hybrids (Fig. 3). Only one microsporocyte showed 17 bivalents and two univalents (Fig. 4); however, the proximity between the univalents in that cell suggests

Table 2. Seed width mean comparisons (mm) for parent Kanlow (K) and Summer (S) plants and for their hybrids' progeny and associated *t* tests assuming unequal variances. *N* = 45 for parents and *n* = 105 for seeds from their hybrids' progeny.

Genotype	Mean	Standard deviation	SE	*t*	*P* > *t*
Kanlow	1.13	0.08	0.012		
Summer	1.18	0.09	0.014	−2.54†	0.01*
S × K	1.17	0.07	0.007	−3.25†	0.01**
K × S	1.12	0.07	0.007	0.31†	0.75ns
S × K	1.17	0.07	0.007	0.20‡	0.86ns
K × S	1.12	0.07	0.007	−3.64‡	0.01**
K × S	1.12	0.07	0.007	−5.00§	0.01**

* Indicates significance at *P* = 0.05.
** Indicates significance at *P* = 0.01.
† Mean comparison with Kanlow.
‡ Mean comparison with Summer.
§ Mean comparison with S × K.

Table 3. Meiotic pairing behavior in upland × lowland tetraploid hybrids of switchgrass.

Hybrid	Observed plants	Cells observed	Chromosome pairs	
			Bivalents	Univalents
	n	*n*		
K1 × S7	10	53	18	0
		1	17†	1
K25 × S8	5	34	18	0
K16 × S30	1	56	18	0
S7 × K1	1	15	18	0
S30 × K16	3	3	18	0

† Shown in Fig. 4.

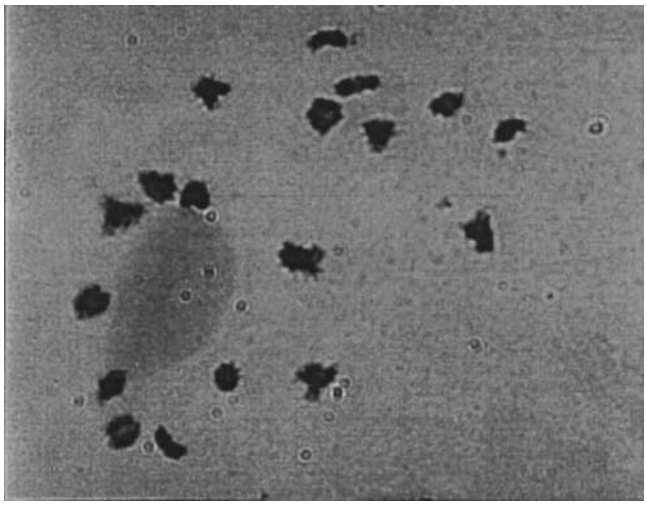

Fig. 3. Microsporocyte of Kanlow by Summer hybrid showing 18 bivalent pairs at diakinesis stage of meiosis.

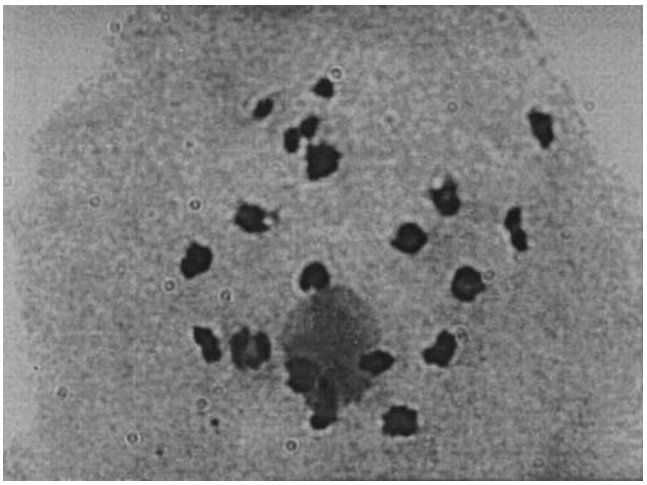

Fig. 4. Microsporocyte of Kanlow by Summer hybrid showing 17 bivalents and two univalents at diakinesis stage of meiosis.

that this disturbance may have resulted from the squashing procedure. The meiotic pairing behavior of the intercytotype switchgrass hybrids is similar to that reported by Lu (1998) and by Barnett and Carver (1967) in tetraploid switchgrass.

The bivalent pairing observed in the hybrids indicates that there is a high degree of homology between chromosomes of the upland and lowland genomes; thus, the transfer of genes between cytotypes likely is possible by recombination resulting from meiotic pairing between homologous chromosome regions. The degree of chromosome pairing also indicates that these two cytotypes are closely related since parents with similar genomes exhibit complete or almost complete chromosome pairing in their hybrids (Singh, 1993).

Hybridization of DNA from the lowland and upland cultivars with the specific spinach cpDNA clone used in this study confirmed the previous findings by Hultquist et al. (1996) of a *Bam*HI site polymorphism between the two ecotypes. The 13-kb fragment was hybridized in upland parents (Lanes A and I) while lowland parents (Lanes B, H, and O) lacked this fragment (Fig. 5). Only the 4.4-kb fragment was detected with the *rbcL*-specific clone pRR12 in lowland genotypes. This result indicates that the polymorphic *Bam*HI restriction site is outside of the *rbcL* region and thus the 8.6-kb fragment detected by Hultquist et al. (1996) using the sorghum probe pLD5 is not detected.

The restriction fragment hybridization pattern detected with the spinach pRR12 cpDNA clone in the progeny Lanes C to F and J to N was the same as that observed in the maternal parent of those progenies (Lanes A and H). For the progeny P to R the upland female parent was not available and only the male lowland parent was evaluated (O). The restriction fragment detected in the progeny Lanes P to R was the same observed in the upland parents. The seedling, whose restriction fragment is shown in Lane S and had the Kanlow restriction fragment, died before its hybrid origin could be verified by morphological markers. We suspect that it was a Kanlow or Kanlow × Summer contaminate. Detection limits of the Southern Blotting system do no rule out the possibility of some paternal

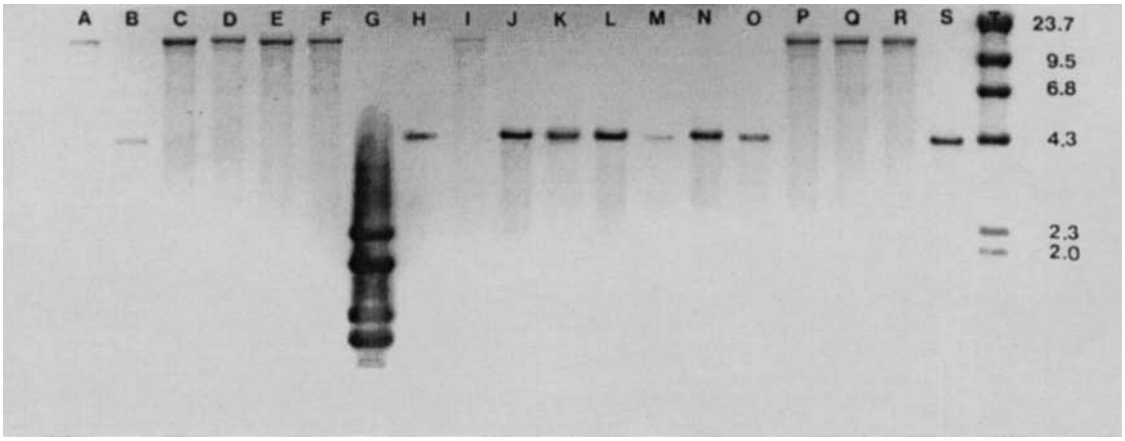

Fig. 5. Chloroplast DNA polymorphism among parents and hybrids of switchgrass. DNA was digested with *Bam*HI restriction endonuclease and hybridized with the spinach cpDNA probe pRR12. Lanes A and B are the Summer female and Kanlow male parents, respectively, of the progeny in Lanes C to F. Lanes H and I are the Kanlow female and Summer male parents, respectively, of the progeny in Lanes J to N. Lane O is the Kanlow male parent of the progeny in Lanes P to R. Lane S could not be verified due to death of the source plant. In the last lane on the right the molecular weight of polymorphic bands of *Hin*dIII-cut λ DNA are marked in kilobases (kb).

transmission of cpDNA. The results here, however, are consistent with the predominant maternal inheritance of cpDNA observed in most of the angiosperm species (Sears, 1980).

In summary, all morphological traits evaluated except seed width were useful to verify the hybrid origin of the seed obtained in the direct and reciprocal crosses made between Kanlow and Summer. The viability of the hybrid seed and the normal meiotic chromosome pairing shown by the hybrids indicate a high degree of similarity between upland and lowland genomes. A maternal inheritance mode of cpDNA was observed in both ecotypes. These results demonstrate that the morphological and genetic differences between the lowland and upland ecotypes are not sufficient to make any other taxonomic distinction between them.

REFERENCES

Alderson, J., and W.C. Sharp. 1955. Grass varieties in the United States. USDA. Lewis Publishers, New York.

Barnett, F.L., and R.F. Carver. 1967. Meiosis and pollen stainability in switchgrass, *Panicum virgatum* L. Crop Sci. 7:301–304.

Brunken, J.N., and J.R. Estes. 1975. Cytological and morphological variation in *Panicum virgatum* L. Southwest Nat. 19:379–385.

Feinberg, A.P., and B.Vogelstein. 1983. A technique for radiolabeling DNA restriction endonuclease fragments with high specific activity. Anal. Biochem. 132:6–13.

Gunter, L.E., G.A. Tuskan, and S.D. Wullschleger. 1996. Diversity of switchgrass based on RAPD markers. Crop Sci. 36:1017–1022.

Hitchcock, A.S. 1971. Manual of grasses of the United States. Vol. II. Dover Publications Inc., New York.

Hopkins, A.A., Ch. M. Taliaferro, C.D. Murphy, and D'Ann Christian. 1996. Chromosome number and nuclear DNA content of several switchgrass populations. Crop Sci. 36:1192–1195.

Hultquist, S.J., K.P. Vogel, D.J. Lee, K. Arumuganathan, and S. Kaeppler. 1996. Chloroplast DNA and nuclear DNA content variations among cultivars of switchgrass, *Panicum virgatum* L. Crop Sci. 36: 1049–1052.

Lu, K., S.M. Kaeppler, K.P. Vogel, K. Arumuganathan, and D.J. Lee. 1998. Nuclear DNA content and chromosome numbers in switchgrass. Great Plains Res. 8:269–280.

Lynd, L.R., J.R. Cushman, R.J. Nichols, and C.E. Wyman. 1991. Fuel ethanol from cellulosic biomass. Science 251:1318–1323.

Martínez-Reyna, J.M., and K.P. Vogel. 1998. Controlled hybridization technique for switchgrass. Crop Sci. 38:876–878.

Moser, L.E., and K.P. Vogel. 1995. Switchgrass, big bluestem, and indiangrass. p. 409–420. *In* R.F Barnes et al. (ed.). Forages, 5th ed. Vol. I: An introduction to grassland agriculture. Iowa State Univ. Press, Ames, IA.

Muza, F.R., D.J. Lee, D.J. Andrews, and S.C. Gupta. 1995. Mitochondrial DNA variation in finger millet [*Eleusine coracana* (L.) Gaerth.]. Euphytica 111:199–205.

Porter, C.L. 1966. An analysis of variation between upland and lowland switchgrass *Panicum virgatum* L. in central Oklahoma. Ecology 47:980–992.

Reed, K.C., and D.A. Mann. 1985. Rapid transfer of DNA from agarose gels to nylon membranes. Nucleic Acids Res. 13:7202–7221.

Riley, R.D., and K.P. Vogel. 1982. Chromosome numbers of released cultivars of switchgrass, indiangrass, big bluestem, and sand bluestem. Crop Sci. 22:1082–1083.

Saghai Maroof, M.A., K.M. Soliman, R.A. Jorgensen, and R. W. Allard. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location, and population dynamics. Proc. Natl. Acad. Sci. (USA) 81:8014–8018.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sears, B.B. 1980. Elimination of plastids during spermatogenesis and fertilization in the plant kingdom. Plastid 4:233–255.

Singh, R.J. 1993. Plant cytogenetics. CRC Press, Boca Raton, FL.

Smith, L. 1947. The acetocarmine smear technique. Stain Techol. 22:17–31.

Vogel, K.P. 1996. Energy production from forage or American agriculture-back to the future. J. Soil Water Conserv. 51:137–139.

Zurawski, G., B. Perrot, W. Bottonley, and P. Whitfeld. 1981. The structure of the gene for the large subunit of ribulose 1,5-bisphosphate carboxylase from spinach chloroplast DNA. Nucleic Acids Res. 9:3251–3270.